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Biosynthesis of Choline & Its Incorporation by Resting Cells of *Neurospora crassa*

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Attempts have been made to demonstrate the synthesis of choline by respiring mycelia of *N. crassa* (wild) from ethanolamine and various methyl precursors under varied experimental conditions. In no case a net increase in choline content has been observed. The ability of the cells to bring about the synthesis could be inferred from the presence of this metabolite in the mycelium harvested from the cholineless medium. More directly the ability to incorporate C^{14} from ($C^{14}H_3$) methionine or (3-C^{14}) serine has been demonstrated.

Mycelia of both the wild type and the cholineless strains can rapidly incorporate choline into the cells, the amount incorporated increasing with the concentration of choline in the suspension medium up to 50 μg./ml. A balance sheet reveals that during the 4 hr period of incubation much (up to 30 per cent) of the choline was lost in unaccountable ways. This is not due to oxidation, demethylation, conversion to trimethylamine or degradations leading to liberation of free ammonia.

THE cholineless mutant strain No. 34486 of *Neurospora crassa*[1] is known to respond to dimethylethanolamine (DMEA) and less markedly to monomethylethanolamine (MMEA)[2], indicating that synthesis of choline in this organism involves the stepwise methylation of ethanolamine and that this strain has a genetic block at the first step, viz. the methylation of ethanolamine to MMEA. Using double mutant strains of *N. crassa*, Nyc[3] has shown that formate is a more direct precursor of the methyl moiety of MMEA than methionine.

The present work deals, in part, with attempts at a more direct demonstration of choline synthesis by resting cells of *N. crassa* from various likely precursors.

Horowitz and Beadle[1] have suggested that formation of lecithin is one of the functions of choline in *Neurospora*, since the former was also active for growth of strain No. 34486. It was, therefore, also of interest to study the incorporation of choline into resting cells of the microorganism.

Methods

The wild type[1] and the cholineless mutant strain No. 34486 of *N. crassa* were grown in 50 ml. Erlenmeyer flasks on 10 ml. of the basal choline assay medium of Horowitz and Beadle[1], and containing 10 μg. choline respectively, for 60 hr at 30°C.

Study of net synthesis of choline — Washed mycelial pads of *N. crassa* (wild), grown as above, were suspended in reaction mixture which, in initial experiments, contained: $10^{-2}M$ ethanolamine, $10^{-2}M$ 1-C precursor, $0{\cdot}1M$ sodium succinate and $0{\cdot}1M$ potassium phosphate buffer (*p*H 7·4), in a total volume of 10 ml. Variations in the system, in subsequent experiments, are indicated in the text. Incubations were carried out at 37°C. At the end of the incubation period, the flasks were steamed for 10 min. to stop the reaction and total choline in the system estimated as described below. In each experiment a control was kept in which the complete reaction system was steamed for 10 min. at zero time.

Dry weight of mycelial matter was determined by weighing mycelial pads grown under identical conditions as those used in the incubation system. The average weight of four pads was taken in each experiment.

Estimation of choline — Mycelial pads were disintegrated in water in a Potter-Elvehjem glass homogenizer and free choline was extracted by heating the aqueous suspension for 1 hr at 80°C. For determination of total choline, the mycelial pad was disintegrated and autoclaved with 3 per cent (vol./vol.) sulphuric acid at 15 lb./sq. in. for 2 hr. After cooling, the mixture was neutralized to congo red with barium hydroxide and the barium sulphate precipitate centrifuged off. Choline in the clear supernatant was adsorbed on activated Decalso columns[4], washed with 5 ml. of 0·3 per cent (wt/vol.) sodium chloride and eluted by 10 ml. of 5 per cent (wt/vol.) sodium chloride. Total choline in the eluate was then assayed microbiologically according to the method of Horowitz and Beadle[1].

Incorporation of activity from labelled 1-*C donors into choline* — *N. crassa* (wild) mycelial pads, grown as before, were incubated at 37°C. in a reaction mixture consisting of $10^{-2}M$ DMEA, $10^{-2}M$ ($C^{14}H_3$) methionine or $10^{-2}M$ (3-C^{14}) serine, 0·1*M* sodium succinate and 0·1*M* potassium phosphate buffer (*p*H 7·4). At the end of the incubation period, the reaction was stopped by steaming for 10 min.

Isolation of total choline, liberated as described earlier, was brought about by a column chromatographic method essentially similar to that adopted by Pilgeram *et al.*[5]. Shorter columns (8 mm. × 30 cm.) of Dowex-50 resin (200 mesh, H form) were employed. Inactive choline (1 mg.) was added as carrier to the autoclaved solution which was adsorbed on the column and eluted with 1·5*N* hydrochloric acid in 10 ml. fractions. Choline was eluted in the 200-240 ml. fractions which were completely free of serine or methionine. The choline-containing fractions were plated on pyrex glass planchets and radioactivity determined employing the Tracerlab-SC-16 windowless gas flow counter in conjunction with a Tracerlab-SC-51 autoscaler. Total activity in the system, before incubation and after disintegration of pads, was determined by plating aliquots on glass planchets and determining radioactivity directly, as described above.

Choline uptake by N. crassa — Mycelial pads, grown for 60 hr, of either the wild type or cholineless mutant of *N. crassa* were incubated with 0·1*M* potassium phosphate buffer (*p*H 7·4), 0·1*M* sodium succinate and varying concentrations of choline. After incubation for 4 hr at 37°C., the mycelial pads were removed from the system and washed thrice with distilled water. Total choline was estimated in the pads as well as in the combined reaction fluid and washings.

The results, in each case, represent a minimum of four independent determinations.

Determination of choline oxidase activity — The activity was estimated manometrically, in a system containing 0·6 ml. of 0·5*M* potassium phosphate buffer (*p*H 7·4), 2 ml. of mycelial suspension, 0·3 ml. of 1*M* sodium succinate and 0·1 ml. of 2 per cent (wt/vol.) choline chloride. Oxygen uptake was determined at regular intervals for a period of 80 min.

Paper chromatographic detection of ethanolamine — The suspension medium in which choline uptake by the preformed mycelium of *N. crassa* (wild) was studied, was lyophilized after removal of the mycelial pad. The residue was redissolved in 0·2 to 0·3 ml. of water and spotted on circular paper chromatograms. The technique was similar to that of Giri *et al.*[6] with butanol-acetic acid-water (40: 10: 50) as solvent and 0·1 per cent (wt/vol.) ninhydrin in acetone as spotting agent. The R_f value of ethanolamine was 0·75.

Estimation of ammonia liberated during incubation — Liberation of ammonia was studied in a system consisting of 0·1*M* potassium phosphate buffer (*p*H 7·4), 0·1*M* sodium succinate, varying concentrations of choline, and disintegrated mycelium equivalent to about 10 mg. dry weight in a total volume of 2 ml. in Conway microdiffusion units[7]. Ammonia liberated was absorbed in 1 ml. of *N*/500 hydrochloric acid and estimated by direct nesslerization after suitable dilution, as described by Umbreit *et al.*[8].

Results

Choline biosynthesis — No net increase in total choline in the system over the zero time control could be observed in incubation of mycelial pads of *N. crassa* (wild) with ethanolamine either with or without methionine or serine as 1-C donors. Presence or absence of succinate, variation of *p*H between 6·0 and 8·0 and time of incubation from 2 to 6 hr had no effect. Since the test organism used for choline assay, *N. crassa* (cholineless), also responds to MMEA and DMEA[2], these more direct precursors could not be employed in lieu of ethanolamine.

The possibility that synthesized choline was oxidized during incubation was investigated by manometrically estimating choline oxidation in a system similar to that used for observing net synthesis. No increase over the endogenous respiration could be observed in the presence of choline.

Addition of ethanolamine, MMEA and DMEA (each at a concentration of 20 μg./ml.) to the growth medium, either in the presence or absence of

TABLE 1 — INCORPORATION OF C^{14} FROM 1-C PRECURSORS INTO CHOLINE BY *N. CRASSA* (WILD)

1-C precursor	Incubation period *hr*	Activity *counts/min./flask* Initial	Final	In isolated choline
($C^{14}H_3$) methionine	2	$5{\cdot}8\times10^5$	$4{\cdot}4\times10^5$	840
	4	$5{\cdot}2\times10^5$	$2{\cdot}9\times10^5$	1130
(3-C^{14}) serine	2	$6{\cdot}8\times10^5$	$4{\cdot}4\times10^5$	415
	4	$6{\cdot}4\times10^5$	$3{\cdot}7\times10^5$	520

The reaction mixture contained: DMEA, $10^{-2}M$; 1-C precursor, $10^{-2}M$; sodium succinate, $0{\cdot}1M$; potassium phosphate buffer (*p*H 7·4), $0{\cdot}1M$; and mycelial pad, 25 mg. dry weight, in a total volume of 10 ml. Incubations were carried out at 37°C.

TABLE 2 — INCORPORATION OF CHOLINE INTO MYCELIA OF *N. CRASSA* (WILD)

Choline added to reaction mixture *μg./flask*	Choline in reaction mixture after incubation *μg./flask*	Choline uptake by mycelial pads *μg./mg. dry wt*	Choline unaccounted for *μg./flask*	Choline unaccounted for %
25	6·5±1·2*	0·50±0·12	6·3±0·2	25·2
50	14·0±2·4	1·11±0·25	10·7±1·3	21·4
75	12·2±2·9	0·97±0·26	13·7±1·8	18·0
100	9·4±2·5	0·86±0·17	16·5±3·3	16·5

The reaction mixture contained: potassium phosphate buffer (*p*H 7·4), $0{\cdot}1M$; sodium succinate, $0{\cdot}1M$; and mycelial pad, 15-25 mg. dry weight in a total volume of 10 ml. Incubations were carried out for 4 hr at 37°C.
*Standard error of the mean.

methionine or serine or formate as 1-C donors (each at a concentration of 10 μg./ml.), with or without folic acid (0·1 μg./ml.), did not, in any way, affect the growth of *N. crassa* (wild) or its choline content which was found to be, on an average, 2·0 μg./mg. mycelial dry matter.

Since net choline synthesis could not be demonstrated, either in resting cells or during growth of the microorganism, the incorporation of the methyl group of methionine or the hydroxymethyl group of serine into choline, on incubation of wild type *N. crassa* mycelium with DMEA and ($C^{14}H_3$) methionine or (3-C^{14}) serine, was studied. In Table 1 are given the results of this experiment. A greater activity was incorporated into choline from methionine than from serine. The total activity in the flask decreased considerably during incubation in both cases, probably due to oxidative loss of labelled carbon.

Choline incorporation into phospholipids — The cholineless mutant of *N. crassa* takes up choline from the suspending medium quite readily. The wild strain also takes up choline even though this does not influence its growth rate. The nature of this uptake was next studied. It was observed that the uptake by both wild type (Table 2) and the cholineless mutant (Table 3) increases with increasing concentrations of choline in the incubation system, reaching a maximum at a choline concentration of 50 μg./flask, beyond which it tends to remain constant. The choline uptake by the mycelium could not account completely for the decrease of choline in the suspension medium. An oxidizable substrate, succinate, was necessary for optimal incorporation, as its omission reduced choline uptake by wild type mycelium, on incubation with 50 μg. choline, from 1·11 to 0·44 μg./mg. dry mycelial matter. Free choline content of the mycelial pads was found to be independent of the concentration of choline in the incubation system.

No stimulation of choline uptake could be obtained by increasing the microorganism's need for choline, since no change was observed in the choline incorporated per mg. dry mycelial matter, whether *N. crassa* (cholineless) was grown in a medium containing optimal or half the optimal concentration of choline. On incubation with 50 μg. of choline uptake by cells grown in 0·5 and 1·0 μg. of choline/ml. of growth medium was, on an average, 0·31 and 0·32 μg./mg. dry mycelial matter respectively.

Effect of inhibitors — As the need for succinate in the incubation system indicated energy requirement for choline incorporation, the effects of inhibitors of

TABLE 3 — INCORPORATION OF CHOLINE INTO MYCELIA OF *N. CRASSA* (CHOLINELESS)

Choline added to reaction mixture *μg./flask*	Choline in reaction mixture after incubation *μg./flask*	Choline uptake by mycelial pads *μg./mg. dry wt*	Choline unaccounted for *μg./flask*	Choline unaccounted for %
25	4·0±0·9*	0·43±0·11	3·0±0·2	12·0
50	7·5±0·9	0·74±0·13	7·5±0·3	15·0
75	8·0±1·4	0·76±0·11	6·0±4·5	8·0
100	7·0±1·6	0·73±0·24	18·5±1·1	18·5

The reaction mixture contained: potassium phosphate buffer (*p*H 7·4), $0{\cdot}1M$; sodium succinate, $0{\cdot}1M$; and mycelial pad 15-25 mg. dry weight in a total volume of 10 ml. Incubations were carried out for 4 hr at 37°C.
*Standard error of the mean.

TABLE 4 — INHIBITION OF CHOLINE UPTAKE BY POTASSIUM CYANIDE AND DINITROPHENOL

Choline added *μg./flask*	Choline uptake by mycelial pads *μg./mg. dry wt* Control	With KCN	With DNP	Inhibition (%) by KCN	DNP
25	0·50±0·12*	0·21±0·08	0·38±0·07	58·1	24·0
50	1·11±0·25	0·41±0·05	0·52±0·05	63·1	53·2
75	0·97±0·26	0·38±0·05	0·53±0·08	60·8	45·4
100	0·86±0·17	0·36±0·11	0·56±0·23	58·1	34·7

$3\times10^{-3}M$ KCN and $10^{-3}M$ DNP were added where indicated. The reaction mixture contained: potassium phosphate buffer (*p*H 7·4), $0{\cdot}1M$; sodium succinate, $0{\cdot}1M$; and mycelial pad, 15-25 mg. dry weight in a total volume of 10 ml. Incubations were carried out for 4 hr at 37°C.
*Standard error of the mean.

respiration and of oxidative phosphorylation were studied. The presence of $3 \times 10^{-3}M$ potassium cyanide in the incubation system considerably decreased choline uptake; $10^{-3}M$ dinitrophenol (DNP) had a similar, though less pronounced, effect (Table 4). In both cases, however, there is still a decrease of choline in the suspension fluid which is not accountable for by mycelial uptake of choline.

Effect of cell rupture and ageing — Rupture of *N. crassa* (wild) cells by grinding with alumina resulted in a total loss of ability of the particulate matter to take up choline from the incubation system.

Ageing of mycelial pads of the microorganism in $0{\cdot}1M$ potassium phosphate buffer (pH 7·4) for periods up to 3 hr did not affect choline incorporation. Ageing overnight, however, almost completely prevented choline incorporation. Ability of the organism to incorporate choline was not restored on addition of the ageing medium or the heated cell-free extract of *N. crassa* (wild) prepared by alternate freezing and thawing of the mycelia, previously disrupted in a Potter-Elvehjem glass homogenizer.

Loss of choline in incubation system — In an attempt to account for decrease of total choline in the incubation system, various possible products of choline catabolism were tested for in the reaction fluid. No ethanolamine could be detected in the concentrated reaction fluid, indicating that choline is not being demethylated to ethanolamine during incubation. Trimethylamine (TMA), when estimated according to the procedure of Dyer[9], was also not present in measurable quantities in the reaction fluid after incubation, either with or without choline. Loss of choline is not apparently associated with degradations resulting in liberation of free ammonia since addition of choline does not further increase the liberation, which was found to be 26·0 μg. ammonia-nitrogen by mycelium (equivalent to 12 mg. dry weight) in a total volume of 2 ml. on incubation for 4 hr at 37°C.

Discussion

That wild type *N. crassa* is capable of synthesizing choline can be inferred from the detection of choline in cells grown in the absence of choline, as well as from the fact that a genetic block renders it incapable of growth in the absence of choline[1]. However, a net synthesis of choline could not be observed on incubation of respiring cells under various conditions with ethanolamine, and the probable 1-C donors, methionine or serine, or on the addition of these precursors to the growth medium. This could not be explained on the basis of oxidation of the choline formed to betaine, since no choline oxidase activity was detected in the microorganism. This may be analogous to the reported absence of demonstrable citrovorum factor synthesis from folic acid even though the former is known to be present in *Neurospora* mycelium[10].

On incubation with DMEA, the immediate precursor of choline and either ($C^{14}H_3$) methionine or (3-C^{14}) serine, incorporation of activity into the isolated choline was observed (Table 1). This incorporation, after 4 hr incubation, was, however, only 0·22 per cent of initial activity in the case of methionine and 0·08 per cent in the case of serine. This relatively low synthesis may explain the inability to detect net increase in choline synthesis.

The absence of any change in the mycelial-free choline content on incubation, with varying concentrations of choline, indicated that the choline taken up must be in the bound form, probably incorporated into the phospholipids. Incorporation of choline into phospholipids involves its preliminary phosphorylation to phosphorylcholine at the expense of adenosine triphosphate[11]. The observation that the addition of DNP partially suppresses uptake further substantiates the possibility that the choline incorporated into the mycelium goes towards formation of phospholipids. It is also possible that the uptake itself is a biochemical process requiring energy as distinct from mere diffusion.

The decrease in total choline in the reaction system on incubation could not be explained as due to oxidation of choline since no choline oxidase could be detected in the organism. Possible reaction products of choline, viz. ethanolamine or TMA, were tested for and found to be absent. Ammonia was liberated during incubation but addition of choline to the system did not increase the amount of ammonia liberated.

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Succinoxidase Activity in Rat Gonads after Cadmium Chloride Administration

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A single subcutaneous injection of cadmium chloride (1 mg./100 g. body weight) causes marked inhibition of succinoxidase activity in the rat gonads. In the testis, the activity of the enzyme is progressively diminished till the 16th day after which there is a relative increase. The ovarian enzyme activity is depressed on the second day of cadmium chloride injection and, after a relative increase on the fourth day, tends to be inhibited again on the seventh day. *In vitro* addition of the salt to gonadal homogenates also depresses enzyme activity. The bearing of these findings on the mechanism of action of cadmium on the gonads is discussed.

ACUTE necrotic changes have been reported in the rat gonads after administration of cadmium chloride[1-3]. In males, the germinal epithelium is irreversibly destroyed but the interstitial elements regenerate after an initial phase of atrophy. The ovarian changes, on the other hand, are transitory[1]. The biochemical and physiological pathways through which cadmium influences the gonads are largely unknown. However, from the nature of vascular changes it has been postulated that anoxia may be an important contributory factor[1,2]. Studies on enzymes concerned with oxygen and hydrogen transport to the gonads have also been suggested in this connection[2]. An inhibitory effect of cadmium on succinic dehydrogenase activity in some tissues (other than gonads) has actually been reported[4,5]; a depression of testicular phosphatase and non-specific esterase activity after cadmium administration is also on record[2,6].

In view of the above an attempt has been made to investigate succinoxidase activity in the rat gonads at different time intervals after administration of cadmium chloride.

Experimental procedure

Colony-bred adult male (150-170 g.) and prepuberal female albino rats (4 to 6 weeks old with vaginal opening closed; 30-45 g.) were sacrificed at different time intervals after a single subcutaneous injection of cadmium chloride (1 mg./100 g. body weight). Succinoxidase activity was measured by the conventional manometric method in aliquots of testicular and ovarian homogenates in Kreb's Ringer phosphate buffer[7]. Aliquots of these homogenates were used to determine the dry weight of the gonads.

The testis and the ovary were examined histologically at different time intervals after cadmium chloride administration and the degenerative (and regenerative) changes described previously were confirmed[1-3].

Results

Testis — Succinoxidase activity of the testis at any particular time interval after cadmium chloride administration was significantly low in comparison to that of the controls ($P < 0.001$). However, the general pattern was a progressive decline in enzyme activity till the 16th day after which there was a relative increase (Table 1; Fig. 1). The maximum inhibition (93 per cent) was recorded on the 16th day. Although the enzyme activity tended to recover after this period the figures never attained even the 2 days' value which continued to remain significantly higher ($P < 0.001$). Cadmium chloride did not exert any comparable effect on the moisture content of the testis. However, between 9 and 90 days after administration of the salt a diminished trend in moisture content was evident; the lowest percentage was recorded on the 30th day (Table 1).

In vitro addition of cadmium chloride to testicular homogenates (167 μg. $CdCl_2$/ml.) caused about 68 per cent inhibition of succinoxidase activity.

Ovary — In case of the ovary, succinoxidase activity was significantly inhibited from 2 days onwards ($P < 0.02$-0.001); the maximum inhibition (55·4 per cent) was noticed on the 7th day (Table 1; Fig. 1).

TABLE 1 — SUCCINOXIDASE ACTIVITY OF RAT GONADS AFTER CADMIUM CHLORIDE ADMINISTRATION

Days after $CdCl_2$ administration	Mean succinoxidase activity (QO_2) with S.E.		Mean moisture content (%)	
	Testis	Ovary	Testis	Ovary
0 (normal controls)	5·76±0·28 (5)*	8·53±0·33 (6)†	87·0 (5)	78 (6)
1	—	8·60±0·60 (6)	—	77 (6)
2	3·40±0·45 (5)	5·07±1·22 (6)	87·0 (5)	79 (6)
4	—	6·89±0·44 (6)	—	79 (6)
7	—	3·80±0·46 (6)	—	66 (6)
9	1·13±0·15 (5)	—	80·5 (5)	—
16	0·41±0·08 (5)	—	81·0 (5)	—
30	1·38±0·19 (5)	—	72·5 (5)	—
90	1·28±0·07 (5)	—	81·0 (5)	—

*Number of animals.
†Mean of three values, each based on pooled ovaries from two animals.

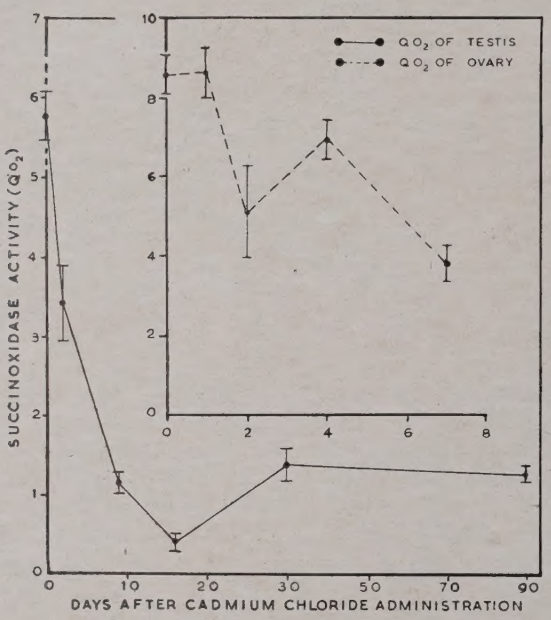

Fig. 1 — Succinoxidase activity of rat gonads after cadmium chloride administrations

However, there was a slight tendency of recovery of the enzyme at 4 days but the value was still significantly lower than that of the controls ($P < 0.02$). The moisture content of the ovary was virtually similar at different time intervals after cadmium chloride administration except at 7 days, when a relatively low value was obtained.

The inhibition of succinoxidase activity after *in vitro* addition of cadmium chloride to ovarian homogenates (167 μg. $CdCl_2$/ml.) was of the same order (68 per cent) as in the testis.

Discussion

The results of the present study indicate that cadmium chloride disturbs the oxidative metabolism of the rat gonads as evident from the nature of alterations in succinoxidase activity. Moreover, there is a correlation between enzymatic and histopathological changes particularly in the case of the testis. Thus, the depression of enzyme activity after cadmium chloride administration is as prompt as the appearance of degenerative changes in the testis; the period of maximum inhibition too is around the phase of greatest destruction of the organ[2,3]. The relative increase in activity of the enzyme after this stage coincides with the regeneration of the interstitial portion of the testis[3]. It must, however, be pointed out that the overall enzyme activity of the organ failed to return to a level comparable to that of the controls even after 90 days of cadmium chloride injection. This may be expected as the seminiferous tubules are permanently destroyed after administration of the salt.

The situation is somewhat anomalous in the females where the maximum inhibition of succinoxidase activity occurs at a time (7 days) when the ovary has recovered fully from the toxic effects of cadmium chloride[1]. Prior to this period, however, the pattern of enzymatic changes correlates with the nature of histopathological events. The destructive changes induced by cadmium chloride reach their acme at 2 days and this is followed by signs of regeneration at 4 days[1]. It is significant that the enzyme activity is otherwise lowest at 2 days but from 4 days there is a relative increase. Perhaps the low moisture content of the ovary at 7 days is a contributory factor towards such anomalous depression of enzyme activity. It is pertinent that the percentage of moisture in the testis registers a fall from 9 days when the inhibition of enzyme activity assumes noteworthy proportions (Table 1). During the earlier period (2 days) the moisture content remains unaltered, and the extent of enzyme inhibition is considerably less. A similar situation exists in the females where the moisture content of the ovary remains virtually unaltered between 1 and 4 days; this period, however, does not coincide with the stage when the enzyme activity is maximally depressed.

The bearing of these findings on the mechanism of action of cadmium on the gonads merits comment. Evidently, the pituitary is not involved at least in the males, because cadmium is able to induce necrotic changes in the testis even in hypophysectomized animals[2]. Any interference with gonadotrophin action at the target level cannot be expected to produce acute degenerative changes of such magnitude in

the gonads[3,8]. A state of deficiency of vitamin E is also not produced by cadmium in the testis[9]. On the other hand, disturbance of oxidative metabolism of the gonads as shown by a consistent inhibition of succinoxidase activity may be of significance in the overall mechanism of cadmium action. Moreover, the *local influence* of the salt on the gonads is borne out by the observation that direct administration into one testis is not followed by destruction of both; the contralateral organ remains normal but the injected one is degenerated in the usual manner[10]. *In vitro* inhibition of succinoxidase activity by cadmium chloride in the gonadal homogenates is also an interesting pointer in this direction.

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Influence of Cobalt on the Synthesis of Vitamin B_{12} in Sewage during Aerobic & Anaerobic Treatment

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A comparative study has been made of the influence of cobalt on the synthesis of vitamin B_{12} in sewage sludges formed under aerobic and anaerobic conditions. The effect of varying the amount of cobalt, from 1 to 50 p.p.m., on the purifying action of activated sludge has also been studied. The experimental evidence on these and related aspects indicated that the conditions in the activated sludge process are more favourable for the synthesis of vitamin B_{12} and that this is facilitated with 1 p.p.m. cobalt added to the sewage. Cobalt at this concentration brings about 50 per cent increase in vitamin B_{12} content of the sludge but does not seem to affect the process of purification or the nitrogen and phosphorus contents of the sludge.

THE occurrence of vitamin B_{12} in activated sludge[1] has evoked considerable interest, particularly in regard to the use of the sludge or its extracts as a source of the vitamin in animal feeds[2,3]. Recent work[4] indicated that, among the sewage sludges examined, activated sludge contained the highest amount of vitamin B_{12}.

In view of the earlier observations on the influence of cobalt on the synthesis of B vitamins[5,6], it was suggested[7] that the vitamin B_{12} content of activated sludge could be increased by the addition of small amounts of cobalt (0·01 to 0·1 p.p.m.) to the sewage in the aeration tank, because such an addition is the usual practice in commercial fermentation for

production of this vitamin. But it was reported that addition of 0·002-40·0 p.p.m. of cobalt did not influence the concentration of this vitamin in activated sludge[8]. Later reports indicated that cobalt chloride stimulated the synthesis of cyanocobalamin and certain B_{12}-like factors in aerobically fermenting sludge[9] and that the vitamin content of the sludge developed from citrus waste increased from 9·15 γ/g. to 24·88 mcg./g. by the addition of 1-2 p.p.m. of cobalt[10].

Thus, the available evidence on the influence of cobalt on the synthesis of vitamin B_{12} in activated sludge is not adequate. Following our recent observations[11], further studies were carried out by simulating the conditions in the activated sludge process and in septic tank. These experiments were run, using sewage from the same source, with and without added cobalt. Apart from investigating the possibility of increasing the vitamin B_{12} content of activated sludge, the effect of varying concentrations of cobalt on the purification of sewage was also examined. The results of these and related studies are discussed in this paper.

Materials and methods

Samples of detritus-free domestic sewage from the sewage works at this Institute were used for the experiments. A solution of cobalt sulphate (chemically pure, $CoSO_4.7H_2O$) was prepared and the concentration of the solution was so adjusted that 1 ml. was equivalent to 1 p.p.m. cobalt. Necessary amounts of this cobalt solution were daily added to samples of sewage to be treated aerobically and anaerobically.

The quality of sewage effluents from the sewage purification systems was determined daily by examining them for turbidity, permanganate values (oxygen absorbed from acidified potassium permanganate solution in 3 min. and 4 hr), dissolved oxygen, cobalt and vitamin B_{12}. The accumulated sludges in the systems were finally analysed for cobalt, vitamin B_{12}, total nitrogen and phosphorus. The sludge from the aerobic system was examined periodically under the microscope for the condition of the dominant organisms.

Turbidity of sewage effluents was determined using Klett-Summerson photoelectric colorimeter with violet filter (420 mμ). The permanganate values and dissolved oxygen were determined by standard methods[12,13]. Nitrogen was determined by the Kjeldahl method. Phosphorus was determined by the method described by Fiske and Subbarow[14] and modified by King[15]. Cobalt was determined colorimetrically[16] as cobalt-nitroso-β-naphthol (a complex formed by the treatment of the samples with α-nitroso-β-naphthol) in chloroform solution, using green filter (540 mμ). Vitamin B_{12} was assayed using *Lactobacillus leichmanii* ATCC 4797 as the test organism[17].

Results

Concentration of cobalt in sewage — Over a period of six weeks, samples of raw sewage were analysed daily except on Sundays. The results of analysis are given in Table 1 along with permanganate values and values for dissolved oxygen and turbidity.

Effect of varying concentration of cobalt on the purification of sewage — It was considered desirable first to study the effect of varying concentrations of added cobalt (1·0 to 50·0 p.p.m.) in the sewage on its

TABLE 1 — ANALYSIS OF SEWAGE SAMPLES

(*Values represent weekly averages*)

Week	Turbidity*	Permanganate value *p.p.m.*		Dissolved oxygen *p.p.m.*	Cobalt† *p.p.m.*
		3 min.	4 hr		
First	150	15	32	nil	0·6
Second	178	15	32	0·2	0·5
Third	146	13	32	0·1	0·5
Fourth	129	12	25	0·1	0·5
Fifth	126	13	21	nil	0·5
Sixth	132	12	25	nil	0·5

*Values for turbidity were determined using Klett-Summerson photoelectric colorimeter with violet filter (420 mμ).
†The cobalt content of the raw sewage samples varied from 0·5 to 0·8 p.p.m.

TABLE 2 — EFFECT OF VARYING COBALT CONCENTRATION ON THE PURIFYING ACTION OF ACTIVATED SLUDGE

(*Sludge used at* 20% *level*; *period of aeration*, 6 *hr*)

Cobalt added *p.p.m.*	Turbidity	Permanganate val. *p.p.m.*		Dissolved oxygen *p.p.m.*	Condition of dominant protozoa†
		3 min.	4 hr		
Control (raw sewage* and sludge)	11	3·0	5·0	6·8	Active
1	12	3·0	5·0	6·6	do
3	27	5·0	13·0	4·6	Inactive
5	33	8·0	15·0	3·2	Dead
7	38	8·0	16·0	3·2	do
10	47	9·0	19·0	2·1	do
15	53	11·0	24·0	0·9	Disintegrated
20	62	13·0	28·0	0·9	do
30	75	15·0	33·0	0·8	do
40	89	18·0	37·0	0·6	do
50	132	22·0	46·0	0·5	do

*The analytical figures for the sewage employed are: turbidity, 129; 3-min. permanganate value, 19·0 p.p.m.; 4-hr permanganate value, 42·0 p.p.m.; dissolved oxygen, 0·1 p.p.m.
†Species of Epistylis and Opercularia.

TABLE 3 — ANALYSIS OF EFFLUENTS FROM AEROBIC TREATMENT OF SEWAGE WITH AND WITHOUT ADDED COBALT

Week	Cobalt in sewage *p.p.m.*		Turbidity		Permanganate val., *p.p.m.*				Dissolved oxygen *p.p.m.*	
					3 min.		4 hr			
	Without Co addition	With added Co	Without Co	With Co	Without Co	With Co	Without Co	With Co	Without Co	With Co
First	0·6	1·5	42	42	5	5	14	14	6·2	6·2
Second	0·3	1·0	17	45	4	4	5	9	6·3	6·3
Third	0·3	0·7	12	50	3	5	5	8	6·6	6·6
Fourth	0·3	0·7	7	21	2	5	5	8	6·7	6·6
Fifth	0·2	0·6	6	8	2	2	5	6	6·8	6·8
Sixth	Trace	0·4	5	5	2	2	4	4	6·8	6·8

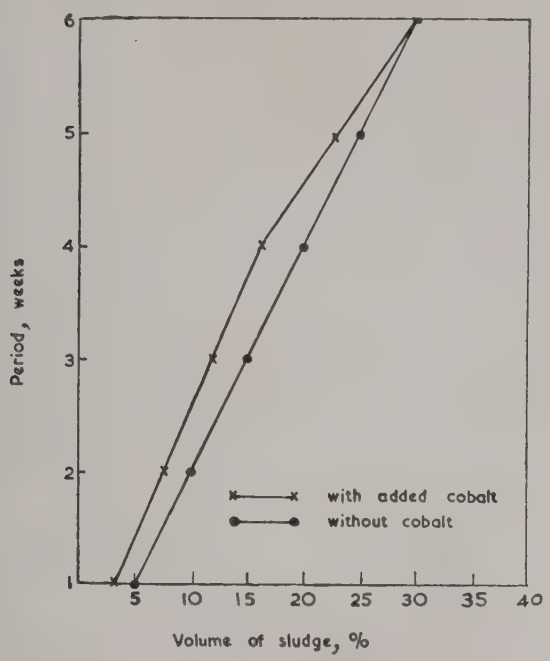

Fig. 1 — Rate of formation of activated sludge with and without added cobalt

purification by activated sludge which would normally show up the effect in a much shorter time than by other methods. So, varying amounts of cobalt were added to samples of sewage, together with a known quantity of activated sludge (20 per cent, by volume), and the mixtures were aerated for 6 hr. The quality of the effluents thus obtained is indicated in Table 2, from which it may be seen that a concentration of cobalt higher than 1 p.p.m. in the sewage adversely affected the purification process to an increasing extent.

Influence of added cobalt on the quality of the effluents and sludges: *Aerobic treatment* — A two-litre sample of the sewage was divided into two equal portions, to one of which was added calculated amount of cobalt sulphate (equivalent to 1 p.p.m. of cobalt). These two one-litre samples were taken in bottles (capacity 1·5 litres) and air was bubbled through the suspensions by means of a suction pump attached to a water tap. The aeration was continued for 72 hr. At the end of this period the clarified supernatant liquids were taken out for analysis, retaining the sludges or deposits formed in the bottles; and the volume of sewage (the volume varied with the accumulation of sludge, from about 980 ml. in the first week to about 700 ml. in the sixth week) equivalent to the liquid taken out was added to each of the bottles and aeration was continued. One of the bottles continued to receive sewage with added cobalt (1 p.p.m.) till the end of the experiment. This operation of ' fill and draw ', which was carried out at 24 hr intervals, was continued over a period of six weeks (with the exception of Sundays). The results of analysis of the effluents taken out daily and the sludges taken out at the end of the experiment are given in Tables 3 and 5. The gradual formation of activated sludge in the two bottles which received sewage with and without added cobalt is indicated in Fig. 1.

Anaerobic treatment — Anaerobic experiments were started at the same time as the aerobic experiments. Two five-litre samples of the same sewage (to one sample cobalt sulphate was added, 1 p.p.m. Co) were taken in two similar aspirator bottles of five-litre capacity. As these bottles were completely filled with sewage, anaerobic conditions were created. After the third day, 500 ml. of sewage were taken out from about the centre of the bottle by means of a suitable bent glass tube attached at the bottom of the bottle and analysed. Sewage (500 ml.) was immediately added to each of the bottles through a separating funnel attached at the mouth of the bottle. As in the aerobic treatment series, the subsequent samplings and additions were made daily; one of the bottles continued to receive sewage containing added cobalt (1 p.p.m.) till the end of the experiment. The results of the daily analysis of the effluents from these anaerobic treatment systems and of the sludges taken

TABLE 4 — ANALYSIS OF EFFLUENTS* FROM ANAEROBIC TREATMENT OF SEWAGE WITH AND WITHOUT ADDED COBALT

Week	Cobalt in sewage *p.p.m.*		Turbidity		Permanganate val., *p.p.m.*				Dissolved oxygen *p.p.m.*	
					3 min.		4 hr			
	Without Co addition	With added Co	Without Co	With Co	Without Co	With Co	Without Co	With Co	Without Co	With Co
First	0·6	1·3	98	98	27	27	53	53	nil	nil
Second	0·4	0·9	97	110	21	25	42	53	nil	nil
Third	0·3	0·9	57	142	14	33	19	55	nil	nil
Fourth	0·4	0·8	53	152	14	32	18	55	nil	nil
Fifth	0·3	0·8	41	128	13	28	15	53	0·3	nil
Sixth	0·3	0·7	38	128	13	24	15	51	0·4	nil

*A week after the start of the experiment the effluents from the bottle containing added cobalt were black, while those from the other bottle were grey; the former effluents gave off more hydrogen sulphide.

TABLE 5 — NITROGEN, PHOSPHORUS AND VITAMIN B_{12} CONTENTS OF SLUDGES

Material	Nitrogen* %	Phosphorus* %	True B_{12} activity† *μg./100 g. of oven-dry material*
Raw sewage solids	3·40	0·48	16·41
Sludge from anaerobic system (septic sludge)			
Without Co	4·03	0·76	9·60
With Co	4·05	0·85	13·30
Sludge from aerobic system (activated sludge)			
Without Co	7·27	1·70	75·40
With Co	6·49	1·68	113·40

*Oven-dry basis.
†Difference between total and alkali-stable activity.

out at the end of the experiment are given in Tables 4 and 5.

Discussion

The raw sewage used for the experiments contained 0·5-0·8 p.p.m. of cobalt. This concentration of cobalt does not apparently affect the treatment of the material either by the activated sludge process or by the septic tank method.

When the concentration of cobalt in the sewage was raised by 1 p.p.m., the quality of the effluent from the aerobic system was slightly affected during the second, third and fourth weeks of the experiment, but became normal during the fifth and the sixth weeks. This variation in the quality of the effluent was reflected on the condition of the sludge, particularly on the volume of the sludge formed. However, the sludge seemed to acclimatize itself gradually over a period of about a month to the added cobalt in the sewage. When the concentration of cobalt was increased beyond 1 p.p.m., its adverse effect on the purifying action of activated sludge was evident; even a concentration of 3 p.p.m. seriously interfered with the process of purification.

The effluent from the anaerobic system receiving sewage with added cobalt (1 p.p.m.) was black, more turbid, less oxygenated and contained more organic matter in comparison with the effluent from the control system without any addition of cobalt. The bottle containing added cobalt, when opened at the end of the experiment, gave off more hydrogen sulphide than the control bottle. These observations indicate that even a concentration of 1 p.p.m. of cobalt in sewage interfered with the operation of septic tank.

The synthesis of vitamin B_{12} in the activated sludge as well as in the septic sludge formed over a period of six weeks with 1 p.p.m. of added cobalt in the sewages was, however, greater than that in the corresponding sludges formed from sewages to which cobalt was not added. It was interesting that in both types of experimental sludge the amounts of vitamin B_{12} were about 1·5 times than those in the corresponding control sludges. But it is not clear how this somewhat uniform increase of the vitamin in the sludges under aerobic and anaerobic conditions was brought about.

The synthesis of vitamin B_{12} during intensive aeration of sewage and formation of activated sludge was, of course, much greater (more than 4·5 times) than that in the raw sewage solids. The septic sludge resulting from samples of the same sewage, however, showed less vitamin than that in the sewage solids. This reduction or loss of vitamin during the formation of septic sludge remains to be investigated.

There is thus clear evidence that activated sludge is a good source of vitamin B_{12}; it has even been considered as one of the richest sources of the vitamin (50 μg./g.)[18]. The experimental evidence now presented would indicate that the vitamin content of the sludge could be appreciably increased by

addition to the sewage of a small amount of cobalt which should not exceed 1 p.p.m.

It is a remarkable feature of activated sludge that it removes from the effluent most of the cobalt ordinarily present in domestic sewage or added to it as in the experiment run for six weeks. In this connection it may be of interest to note that studies carried out some ten years ago on the fate of radioactive materials in sewage treatment, using Co-60, among other isotopes, showed that Co-60 was strongly adsorbed on to the sludge and removed from the effluent to the extent of about 79 per cent[19]. The removal of Co-60 during preliminary sedimentation process was very little[19]. In the present study the behaviour of septic sludge, which may be regarded as a material formed during prolonged sedimentation, is not very different from that reported for the removal of Co-60 during sedimentation.

Addition of cobalt (1 p.p.m.) to the sewage does not appreciably affect the fertilizing value of the sludges; this is evident from the figures for total nitrogen and phosphorus of the sludges. Moreover, it was found that after the extraction of vitamin B_{12} from activated sludge for animal feed enrichment, the fertilizing value of the sludge was left unchanged[3].

The present studies would, therefore, suggest that the vitamin B_{12} content of activated sludge could be appreciably increased by addition to the sewage of a small amount of cobalt, not exceeding 1 p.p.m., and this treatment would not affect the purification process or the concentration of nitrogen and phosphorus in the sludge. The sludge could be directly used as a feed supplement to chicks, pigs, etc., if extraction of the vitamin is not considered necessary.

Summary

Experiments carried out over a period of six weeks with samples of sewage from the same source showed that aerobic treatment as by the activated sludge process was clearly more favourable for the synthesis of vitamin B_{12} in the sludge and that addition of 1 p.p.m. of cobalt to the sewage brought about an appreciable increase of the vitamin in the resulting activated sludge, the increase being about 50 per cent more than that in the sludge developed from sewage without the added cobalt.

Addition of higher amounts of cobalt increasingly affected the microbial activity in the sludge and its purifying action.

Under anaerobic conditions, as in the septic tank, however, addition of even 1 p.p.m. of cobalt had an adverse effect on the quality of the effluent, although the amount of vitamin B_{12} in the sludge was also about 50 per cent more than that in the sludge derived from the sewage without added cobalt.

The significance of these and other observations has been discussed.

Acknowledgement

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Chemotherapy of Tuberculosis: Part VIII—Synthesis of Some New N-Aryl-N′-2-pyrimidylthioureas

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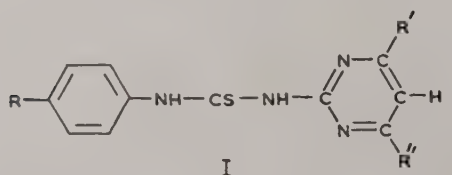
A number of new pyrimidylthioureas have been prepared by the condensation of phenylisothiocyanate or *p*-alkoxyphenylisothiocyanates with 2-aminopyrimidine and its derivatives, and tested for their antitubercular activity. N-Phenyl-N′-2-pyrimidyl and N-phenyl-N′-(4-methyl-6-methoxy)-2-pyrimidylthioureas inhibit the growth of *Mycobacterium tuberculosis* var. *hominis* (strain H37Rv) in a concentration of 1 μg./ml. when tested in Youman's medium.

THIOUREA, and its phenyl and sulphanilyl derivatives display considerable tuberculostatic activity[1]. Of the several hundred thioureas synthesized and tested[2-4] during the last few years, some bearing an alkoxyphenyl group have shown promising activity and N-N′-di-(*p*-isoamyloxyphenyl)-thiourea has been reported to be effective in human leprosy[5] and in mice infected with influenza virus[6]. A few derivatives containing a heterocyclic substituent have also been investigated and 4-*n*-butoxy-4′-α-pyridylthiocarbanilide has been claimed to be twelve times as active as *p*-aminosalicylic acid[4]. Among the N-substituted heterocyclic derivatives, N-5-chloro-2-pyridyl-N′-*p*-fluorophenylthiourea has been reported[7] to possess a very high antiviral activity.

In view of these findings and the fact that sulphapyrimidines possess a high degree of antibacterial activity, we have synthesized a series of pyrimidylthioureas of the formula (I)



I

wherein R is hydrogen or an alkoxy group containing a maximum of four carbon atoms and R′ and R″ represent a hydrogen, a methyl or a methoxy group. Heating an alcoholic solution of the amino compound and the appropriate isothiocyanate, as described by previous workers[2,8], failed to furnish the desired thiourea, and only the corresponding alkoxyphenylthiourethanes could be isolated. Buu Hoi *et al.*[2,7] have also reported that the 2-aminopyrimidines could not be condensed with aryl isothiocyanates. However, the required compounds were then obtained by refluxing the two reactants in toluene for 10-12 hr. Most of these thioureas could be prepared by this procedure with the exception of only two members (R-350 and 358); the condensation product in these cases is always associated with some impurity, not removable even after several recrystallizations. This is probably due to dismutation of the thioureas formed, as a result of prolonged heating at a high temperature[2]. These two derivatives, however, could be obtained in pure form, though in low yields, by refluxing a solution of the two reactants in chloroform for about 40 hr.

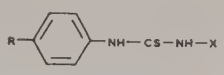
The compounds prepared are listed in Table 1. They were tested *in vitro* against *Mycobacterium tuberculosis* var. *hominis* (strain H37Rv) in Youman's medium, using the serial dilution method. Six compounds (R-354, 360, 362, 365, 368 and 376) showed antitubercular activity in concentrations of 2 μg./ml. and two compounds (R-349 and 373) inhibited the growth in a concentration of 1 μg./ml. Further investigations are under progress and the details will be published elsewhere.

Experimental procedure

2-Aminopyrimidines—2-Aminopyrimidine was prepared by the condensation of β-ethoxyacrolein diethylacetal with guanidine hydrochloride[9]. 2-Amino-4-methylpyrimidine was obtained by the dechlorination of 2-amino-4-methyl-6-chloropyrimidine, using zinc dust in aqueous medium[10]. 2-Amino-4-

*Government of India scholar.
†Unichem scholar.

TABLE 1 — N-ARYL-N'-2-PYRIMIDYLTHIOUREAS



Compound Sl No.	R	M.P. °C.	Mol. formula	Yield %	Nitrogen, % Found	Nitrogen, % Reqd
			X = 2-Pyrimidyl			
R-349	Hydrogen	201-2	$C_{11}H_{10}N_4S$	95	24·31	24·34
R-350	Methoxy	226	$C_{12}H_{12}N_4OS$	76	21·61	21·53
R-351	Ethoxy	221	$C_{13}H_{14}N_4OS$	46	20·93	20·43
R-352	*n*-Propoxy	189-90	$C_{14}H_{16}N_4OS$	26	19·62	19·44
R-353	Isopropoxy	201	$C_{14}H_{16}N_4OS$	52	19·48	19·44
R-354	*n*-Butoxy	179-80	$C_{15}H_{18}N_4OS$	67	18·72	18·54
R-355	Isobutoxy	191	$C_{15}H_{18}N_4OS$	32	18·26	18·54
R-356	*sec*-Butoxy	182	$C_{15}H_{18}N_4OS$	34	18·51	18·54
			X = 4-Methyl-2-pyrimidyl			
R-357	Hydrogen	200	$C_{12}H_{12}N_4S$	41	23·12	22·95
R-358	Methoxy	225	$C_{13}H_{14}N_4OS$	39	19·97	20·43
R-359	Ethoxy	208	$C_{14}H_{16}N_4OS$	31	19·90	19·44
R-360	*n*-Propoxy	179-80	$C_{15}H_{18}N_4OS$	20	18·18	18·54
R-361	Isopropoxy	167-8	$C_{15}H_{18}N_4OS$	50	19·08	18·54
R-362	*n*-Butoxy	186	$C_{16}H_{20}N_4OS$	56	17·55	17·72
R-363	Isobutoxy	177	$C_{16}H_{20}N_4OS$	27	17·15	17·72
R-364	*sec*-Butoxy	167	$C_{16}H_{20}N_4OS$	25	17·44	17·72
			X = 4,6-Dimethyl-2-pyrimidyl			
R-365	Hydrogen	210	$C_{13}H_{14}N_4S$	84	22·06	21·70
R-366	Methoxy	188	$C_{14}H_{16}N_4OS$	68	19·23	19·44
R-367	Ethoxy	178	$C_{15}H_{18}N_4OS$	67	18·59	18·54
R-368	*n*-Propoxy	165	$C_{16}H_{20}N_4OS$	56	17·57	17·72
R-369	Isopropoxy	164	$C_{16}H_{20}N_4OS$	68	17·75	17·72
R-370	*n*-Butoxy	148-9	$C_{17}H_{22}N_4OS$	62	16·79	16·96
R-371	Isobutoxy	163-4	$C_{17}H_{22}N_4OS$	48	16·56	16·96
R-372	*sec*-Butoxy	143-4	$C_{17}H_{22}N_4OS$	48	17·12	16·96
			X = 4-Methyl-6-methoxy-2-pyrimidyl			
R-373	Hydrogen	203	$C_{13}H_{14}N_4OS$	74	20·10	20·43
R-374	Methoxy	201-2	$C_{14}H_{16}N_4O_2S$	61	18·39	18·42
R-375	Ethoxy	194	$C_{15}H_{18}N_4O_2S$	28	17·24	17·61
R-376	*n*-Propoxy	168	$C_{16}H_{20}N_4O_2S$	54	16·90	16·87
R-377	Isopropoxy	178	$C_{16}H_{20}N_4O_2S$	40	16·89	16·87
R-378	*n*-Butoxy	164	$C_{17}H_{22}N_4O_2S$	58	15·85	16·18
R-379	Isobutoxy	168	$C_{17}H_{22}N_4O_2S$	50	15·95	16·18
R-380	*sec*-Butoxy	162-3	$C_{17}H_{22}N_4O_2S$	35	15·85	16·18

methyl-6-hydroxypyrimidine was obtained by the condensation of ethylacetoacetate and guanidine carbonate in the presence of sodium hydroxide in ethyl alcohol[11]. 2-Amino-4,6-dimethylpyrimidine was prepared by the interaction of guanidine carbonate and 2,4-pentanedione[12].

2-Amino-4-methyl-6-methoxypyrimidine — 2-Amino-4-methyl-6-chloropyrimidine (36 g., 0·25 mole) was added with stirring to a hot solution of potassium hydroxide (14 g., 0·25 mole) in methanol (140 ml.). The reaction mixture was refluxed for 2·5 hr, and then filtered hot to remove the potassium chloride formed. The cake was washed with hot methanol and the combined filtrates and washings were concentrated and then diluted with water, when the required compound was thrown out as a colourless solid. This was crystallized from ethylene dichloride; yield 24·2 g. (70 per cent of theory); m.p. 158° (Backer and Grevenstuk[13]).

p-Alkoxyanilines — *p-n*-Propoxy, *p-n*-butoxy and *p*-isobutoxyanilines were prepared in good yields by the reduction of the appropriate *p*-alkoxynitrobenzenes with stannous chloride and hydrochloric acid[14]. All the alkoxynitrobenzenes were obtained in almost theoretical yield by refluxing a solution of *p*-nitrophenol (1 mole) in ethyl cellosolve with anhydrous potassium carbonate (1 mole) and the appropriate alkyl bromide (1·5 moles). *p*-Isopropoxy and *p-sec*-butoxyanilines were, however, prepared by the acid hydrolysis of the corresponding N-acetyl derivatives which were obtained in excellent yields by refluxing, for 6 hr, a mixture of *p*-acetamidophenol

(1 mole), anhydrous potassium carbonate (1 mole) and the appropriate alkyl bromide (1 mole) in ethyl cellosolve.

Aryl isothiocyanates — Phenyl, *p*-anisyl and *p*-phenetylisothiocyanates could be obtained conveniently by following the procedure for phenylisocyanate described by Dains *et al.*[15]. In the case of *p-n*-propoxy, isopropoxy, *n*-butoxy, isobutoxy and *sec*-butoxy phenylisothiocyanates, a similar procedure was adopted but the isothiocyanates were isolated by extracting the dark reaction product with petroleum ether, as these isothiocyanates were not readily steam volatile. All the isothiocyanates were finally purified by fractional distillation under reduced pressure (4-10 mm. Hg).

N-Aryl-N'-2-pyrimidylthioureas (*all compounds except R*-350 *and* 358) — A mixture of the appropriate 2-aminopyrimidine (0·1 mole) and the required arylisothiocyanate (0·1 mole) in toluene (5 ml. for each g. of 2-aminopyrimidine used) was refluxed for 10 to 12 hr, cooled and diluted with petroleum ether. The thiourea was collected, washed well with petroleum ether and then with cold dilute hydrochloric acid, water and dried. All these thiourea derivatives were crystallized from alcohol and had sharp melting points.

N-p-Anisyl-N'-2-pyrimidylthiourea (*R*-350) — A mixture of 2-aminopyrimidine (0·95 g., 0·01 mole) and *p*-anisylisothiocyanate (1·65 g., 0·01 mole) in chloroform (10 ml.) was refluxed for 40 hr. After evaporating off chloroform, the thiourea was isolated as in the above case and was recrystallized from alcohol; m.p. 226°; yield 0·4 g.

N-p-Anisyl-N'-(4-methyl)-2-pyrimidylthiourea (*R*-358) — A solution of 2-amino-4-methylpyrimidine (1·09 g., 0·01 mole) and *p*-anisylisothiocyanate (1·65 g., 0·01 mole) in chloroform (10 ml.) was refluxed for 40 hr. The reaction mixture was then worked up as described above. On recrystallization from ethanol the compound separated as colourless solid; m.p. 225°; yield 0·33 g.

Condensation of p-anisylisothiocyanate with 2-amino-4,6-dimethylpyrimidine in alcohol — A mixture of the isothiocyanate (1·65 g., 0·01 mole) and the pyrimidine (1·23 g., 0·01 mole) in alcohol (15 ml.) was refluxed for a period of 2 hr on a steam bath. The alcohol was evaporated off and the residue extracted with petroleum ether. The insoluble residue (1·1 g.) was found to be the unreacted pyrimidine. The filtrate on concentration furnished a compound which on recrystallization from petroleum ether had m.p. 80°; yield 0·7 g. The product was identified as *p*-anisylthiourethane[16] (mixed melting point with that of an authentic sample not depressed).

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Studies on the Nutritive Value of *Idli* Fortified with Indian Multipurpose Food

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The nutritive value of *idli* (a steamed cake from a fermented dough of rice and black gram *dhal*) fortified with Indian multipurpose food or Bengal gram flour has been studied by growth experiments on albino rats. The results show that (1) unfortified *idli* diet promotes significantly higher growth than rice diet and (2) diet containing *idli* fortified with multipurpose food promotes significantly higher growth than those containing unfortified *idli* or *idli* fortified with Bengal gram flour. The livers of animals receiving the rice diet show parenchymal damage of a mild protein deficiency type, while the livers of animals receiving *idli* or *idli* fortified with Bengal gram and Indian multipurpose food are quite normal. The mean fat content of livers of rats receiving diets based on unfortified *idli* and *idli* fortified with Indian multipurpose food or Bengal gram was significantly less than that of livers of animals fed on the rice diet.

IDLI is a common food preparation widely consumed in many South Indian homes. It is usually prepared by steaming a fermented dough of rice and black gram (*Phaseolus mungo*) *dhal*. *Idli* is generally considered as a nourishing and easily digestible food. Radhakrishna Rao[1] studied the effect of feeding *idli* prepared from fermented and unfermented dough of rice and black gram *dhal* on the liver fat of albino rats. They found that *idli* prepared from fermented dough was more effective in preventing the deposition of fat in the liver than *idli* from unfermented dough. *Idli*, as commonly prepared, has a protein content varying from 11 to 13 per cent on moisture-free basis. Since protein malnutrition is widely prevalent among children belonging to the low income sections of the population, it was felt that *idli* enriched with protein-rich foods will form a suitable supplement for the treatment and prevention of protein malnutrition in children. Purushowtham Rao[2] and Venkatachalam *et al.*[3] have reported that Indian multipurpose food (IMPF) (a 3 : 1 blend of low fat groundnut flour and Bengal gram flour fortified with vitamins and minerals) and Bengal gram *dhal* are quite effective in the treatment of protein malnutrition in children. In the present investigation, the effect of fortification of *idli* dough with Bengal gram and multipurpose food on the nutritive value of *idli* has been studied using albino rats.

Experimental procedure

Idli mixes — The three mixes used in the preparation of *idli* were as follows: Mix I — rice 66·6 per cent + black gram *dhal* 33·3 per cent; mix II — rice 50 per cent + black gram *dhal* 25 per cent + Bengal gram *dhal* 25 per cent; and mix III — rice 50 per cent + black gram *dhal* 25 per cent + IMPF 25 per cent.

The above blends were prepared by mixing the required amounts of coarsely powdered rice and finely powdered *dhals* in a mechanical mixer.

The composition of the Indian multipurpose food used in the present investigation was the same as that described earlier[2]. The three mixes (I, II and III) and the sample of rice used in the preparation of *idli* were analysed for proximate principles and minerals by the methods of the Association of Official Agricultural Chemists[4]. Vitamin A, thiamine and riboflavin contents were determined according to the methods of the Association of Vitamin Chemists[5]. The results are given in Table 1.

Preparation of idli — *Idli* was prepared daily as follows according to the method developed by Desikachar *et al.*[6]. The *idli* mixes (I, II and III) were mixed with about one and a half times the weight of water and made into a smooth dough. Rice flour, which was used as the control, was also made into a dough in the same way. Baker's yeast (20 mg. per cent)

TABLE 1 — CHEMICAL COMPOSITION OF *IDLI* MIXES AND PARBOILED RICE

(*Values per* 100 *g.*)

Constituent	Rice, parboiled (milled)	*Idli* mix I	*Idli* mix II	*Idli* mix III
Moisture, g.	12·50	12·40	12·20	12·30
Protein (N×6·25), g.	6·90	12·10	14·80	19·70
Fat, g.	0·40	0·80	1·90	2·70
Carbohydrate (by diff.), g.	79·10	73·10	69·20	62·20
Ash, g.	0·80	1·60	1·90	3·10
Calcium, g.	0·01	0·07	0·07	0·22
Phosphorus, g.	0·11	0·22	0·25	0·37
Iron, mg.	2·20	4·70	5·80	4·90
Thiamine, mg.	0·21	0·28	0·31	0·54
Riboflavin, mg.	0·10	0·19	0·38	0·89
Vitamin A value, I.U.	—	21	44	766
Vitamin D, I.U.	—	—	—	75

TABLE 2 — PERCENTAGE COMPOSITION OF EXPERIMENTAL DIETS

Rice or *idli* mix	78·5
Tur (*Cajanus cajan*) *dhal*	5·0
Non-leafy vegetables [potatoes and brinjal (*Solanum melongena*)]	8·2
Leafy vegetables (*Amaranthus gangeticus*)	2·1
Groundnut oil	5·0
Skim milk powder	0·9
Common salt	0·3

on the basis of dry weight of flour was suspended in water and added to the dough. A small amount of sour butter milk (as a source of lactic acid bacteria) was added and the dough was allowed to ferment overnight. The dough from the different mixes was transferred, in portions, to small aluminium cups and steamed at atmospheric pressure for 20 min. The resulting *idli* was allowed to cool.

Preparation of experimental diets — The composition of the control poor rice diets was the same as that recommended by the Vanaspathi Research Advisory Committee[7]. The experimental diets (Table 2) were prepared by replacing rice by *idli* prepared from the different blends. The other ingredients of the diets were mixed with three times the weight of water, cooked in steam for 10 min. and mixed with *idli*.

Feeding of experimental animals — Groups of freshly weaned albino rats of the Wistar strain weighing about 40-45 g. (12 in each group and distributed equally according to sex, litter mates and body weight) were fed *ad libitum* on the different diets. The animals were allotted to different groups according to a simple randomized block design, and were housed individually in raised wire screen bottom cages. Another group of weanling rats (6 males weighing about 40 g.) was used for determining the initial body composition. Records of daily food intake were kept for all the groups and the rats were weighed weekly. After feeding for a period of 8 weeks, the haemoglobin and red blood cell count of the animals were determined in the blood drawn from the tails of the rats according to the methods adopted earlier by Joseph *et al.*[8]. The animals were anaesthetized with amytal and the livers were excised after the animals were bled through the abdominal aorta. The excised livers were washed with normal saline to remove any adhering blood, wiped between filter papers and immediately weighed in glass dishes. The livers were analysed for moisture, fat and protein according to the methods of Joseph *et al.*[8].

The contents of the gastro-intestinal tract were removed by squeezing and the whole carcass was mashed in a meat mincer and weighed. The carcass was analysed for moisture, fat and protein according to the procedures used for the analysis of liver.

The initial protein contents of the carcass of the rats in the different groups were calculated from the values obtained for a similar group of weanling rats (6 males) weighing about 40 g. Using this data the retention of protein in the carcass of rats fed on the different diets over a period of 8 weeks was calculated.

Histological examination of liver — Samples of liver taken from the left lobe were fixed in 10 per cent formalin. The tissues were processed through paraffin. Sections of 5 μ thickness were stained with haematoxylin and eosin. In addition, frozen sections were taken from each specimen and stained with

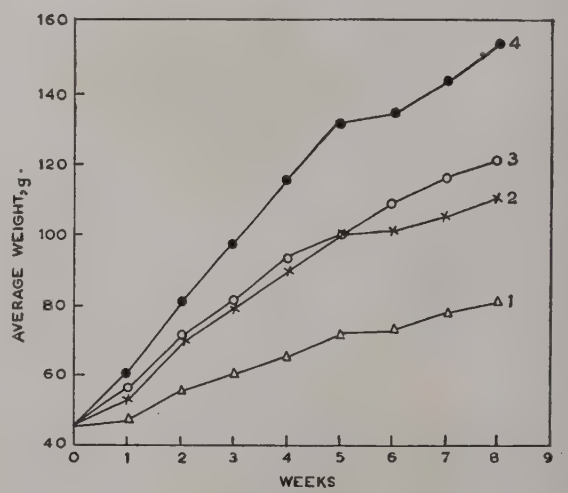

Fig. 1 — Growth rate of rats fed on poor Indian diets based on rice and *idli* fortified with Bengal gram and multipurpose food [(1) Rice; (2) *idli* (unfortified); (3) *idli* fortified with Bengal gram and (4) *idli* fortified with Indian multipurpose food]

Sudan IV in propylene glycol for studying the distribution of fat.

Results

Growth and composition of blood — The rate of growth of animals on the different diets is shown in Fig. 1 and the results obtained in the experiments are shown in Tables 3 and 4. Table 3 shows that the average weekly gain in weight of animals fed on the three *idli* mixes was significantly higher ($P < 0.001$) than that of animals fed on rice diet. *Idli* fortified with IMPF promoted a significantly higher growth rate than unfortified *idli* or *idli* fortified with Bengal gram *dhal*. There was no significant difference in the mean haemoglobin content or R.B.C. count of the blood of the groups of rats fed on the different diets.

Composition of liver and carcass — Table 4 shows that there was no significant difference in the mean protein and moisture contents of the livers of rats fed on the different diets. The mean fat content of livers of rats receiving the diets containing the *idli* mixes I, II and III was, however, significantly lower ($P < 0.001$) than that of the liver of animals receiving the rice diet. The carcass of rats fed on the rice diet contained significantly more moisture and less fat than those of rats receiving the *idli* mixes I, II and III.

Histological structure of liver — The livers of animals fed on the control (rice) diet showed a mild degree of parenchymal damage of the protein deficiency type. No appreciable fatty change or necrosis of the livers was observed. The livers of rats receiving the diets based on unfortified *idli* and *idli* fortified with Bengal gram *dhal* or IMPF were quite normal, showing thereby that the different protein foods were effective in correcting the protein deficiency in the rice diet.

Intake and retention of protein — The intake and retention of protein by the rats fed on the different

TABLE 3 — AVERAGE INCREASE IN BODY WEIGHT AND HAEMOGLOBIN AND RED BLOOD CELL COUNT OF RATS FED ON DIETS BASED ON RICE AND *IDLIS*

Group	Diet	Initial body wt *g.*	Av. daily food intake (dry wt) *g.*	Av. weekly gain in wt *g.*	Haemoglobin* *g./100 ml. blood*	Red blood cell count* 10^6/*cu. mm. blood*
A	Rice	45·2	9·7	4·93±0·38	14·02	8·32
B	*Idli* mix I	45·7	10·1	8·03±0·36	15·08 ±0·24 (15 d.f.)	9·22 ±0·19 (15 d.f.)
C	*Idli* mix II	45·5	10·0	9·44±0·36	15·09	9·21
D	*Idli* mix III	45·6	10·6	13·72±0·32	15·51	9·27
				Test of significance		
	A ~ B			S at 0·1% level	S at 1% level	S at 1% level
	A ~ C			S at 0·1% level	S at 1% level	S at 1% level
	A ~ D			S at 0·1% level	S at 0·1% level	S at 1% level
	B ~ C			S at 5% level	N.S.	N.S.
	B ~ D			S at 0·1% level	N.S.	N.S.
	C ~ D			S at 0·1% level	N.S.	N.S.

*Values are for 6 male rats only.
S, significant, N.S., not significant.

TABLE 4 — COMPOSITION OF LIVER AND CARCASS OF RATS ON DIETS BASED ON RICE AND FORTIFIED *IDLIS*

(*Mean values for groups of 6 males per group for the experimental period of 8 weeks*)

Group	Diet	Body wt *g.*	Fresh wt of liver *g.*	Liver composition: Moisture %	Liver composition: Total protein %	Liver composition: Fat %	Carcass composition: Moisture %	Carcass composition: Total protein %	Carcass composition: Fat %
A	Rice	83·0	3·2	71·37	18·99	4·10	63·34	18·27	13·56
B	*Idli* mix I	114·5	4·1	71·53 ±0·37 (15 d.f.)	19·72 ±0·44 (15 d.f.)	2·98 ±0·11 (15 d.f.)	57·50 ±0·46 (15 d.f.)	19·56 ±0·45 (15 d.f.)	18·35 ±0·45 (15 d.f.)
C	*Idli* mix II	128·5	4·5	71·32	19·86	3·24	57·26	19·86	18·83
D	*Idli* mix III	173·3	6·7	70·01	19·88	3·25	55·60	20·76	19·95
					Test of significance				
	A ~ B			N.S.	N.S.	S at 0·1% level	S at 0·1% level	N.S.	S at 0·1% level
	A ~ C			N.S.	N.S.	S at 0·1% level	S at 0·1% level	S at 5% level	S at 0·1% level
	A ~ D			S at 5% level	N.S.	S at 0·1% level	S at 0·1% level	S at 1% level	S at 0·1% level
	B ~ C			N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	B ~ D			S at 1% level	N.S.	N.S.	S at 1% level	N.S.	S at 5% level
	C ~ D			S at 5% level	N.S.	N.S.	S at 5% level	N.S.	N.S.

S, significant; N.S., not significant.

TABLE 5 — INTAKE AND RETENTION OF PROTEIN IN RATS ON DIETS BASED ON RICE AND FORTIFIED *IDLIS*

(*Mean values for 6 male rats per group; experimental period, 8 weeks*)

	Rice diet	*Idli* mix I	*Idli* mix II	*Idli* mix III
Total protein intake, g.	46·97	77·11	90·88	124·10
Total increase in body wt, g.	37·30	68·30	82·80	127·60
Initial body protein*, g.	8·00	8·08	8·00	8·00
Total body protein, g.	15·17	22·40	25·52	35·97
Protein retained, g.	7·17	14·32	17·32	27·97
Protein retained per 100 g. increase in body wt, g.	19·23†	20·96†	21·15†	21·92†

*Initial body protein content was calculated using the values for the protein content of weanling rats of similar age as described in the text.

†Standard error with degrees of freedom ±0·26 (15 d.f.).

diets are given in Table 5. The results show that the amount of protein retained per 100 g. increase in body weight of rats receiving the diets containing *idli* fortified with IMPF is significantly higher than that of rats receiving the *idli* diet. It is, therefore, evident that the high increase in body weight observed in case of rats fed with *idli* fortified with multipurpose food was followed by a corresponding increase in the protein content of the body.

Discussion

The results obtained in the present investigation have shown that incorporation of IMPF at 25 per cent level in *idli* mix produced a marked improvement in the nutritive value of *idli* as judged by the growth of albino rats. It is of interest to note that incorporation of 25 per cent of Bengal gram *dhal* in the *idli* mix produced significantly less improvement than IMPF. In view of the scarcity and high cost of milk and other protein-rich foods of animal origin in different parts of India, *idli* enriched with IMPF can be used as a cheap and effective source of protein and other dietary essentials for the treatment and prevention of protein malnutrition in children belonging to the low income groups of the population.

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The Role of Phenolic Bodies in the Metabolism of Sandal (*Santalum album* Linn.) in Health & Disease

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The level of phenolic bodies (simpler phenols, polyphenols, tannins and anthocyanins) present in healthy, partially spiked and completely spiked sandal leaves has been determined. The level of phenolic bodies in the leaves of spiked and partially spiked plants has been found to be significantly higher than that in the leaves of healthy plants. This difference is observed even when there is a rapid decrease in the level of phenolic bodies in the leaves as the season advances. The higher level of phenolic bodies in the diseased leaves appears to be a function distinctive of the metabolism of the sandal leaves as it is independent of seasonal effects.

THE role of phenolic bodies (simpler phenols, polyphenols, tannins and anthocyanins) in plant respiration and plant metabolism in general has been discussed by Mason[1]. The literature on the role of phenolic bodies in sandal (*Santalum album* Linn.) metabolism is, however, meagre. Iyengar[2], in a study of the influence of tannins on diastatic activity, recorded higher tannin content in spiked sandal leaves compared to healthy ones. A detailed examination of the total phenolic bodies in the leaves of healthy and spiked sandal plants has not, however, been undertaken so far. Such a study has now been carried out and the results are reported in this paper.

Materials and methods

Sandal trees of approximately the same age (12 to 15 years) were selected for taking leaf samples. Fresh sandal leaves (120-150 g.) collected in the morning (before 9 a.m.) were passed through a meat juice extractor and the resulting fine pulp was squeezed through lawn cloth to get the tissue fluid. The technique employed was such that approximately the same pressure extending over the same period was employed to extract the tissue fluid, starting with comparable weights of leaf material. A known weight of the fluid was suitably diluted (500-2000 times) and the diluted fluid was used for analysis. The actual degree of dilution was governed by the desirability of having approximately the same intensity of colour in the end reaction of estimation. This was determined by preliminary trials. The trees selected for the experimental material were from the nursery of the Forest Research Laboratory, Bangalore; Lal Bagh, Bangalore; Kenchanahally (a forest plantation about 8 miles south-west of Bangalore) and Dobbspet (a forest area about 31 miles north-west of Bangalore). This spread-over incidentally enables us to determine whether any differences exist in the trend of the results for leaves obtained from trees growing in different areas. Also, to study the seasonal effect, the analyses were carried out successively during the months of July, August and September.

After a careful consideration of the pros and cons of available methods for the estimation of phenolic bodies, a colorimetric method based on azo dye formation was selected. The deciding factors were sensitivity and minimum interference by other substances. The reagent selected was Fox and Gauge's diazotized sulphanilic acid (DSA)[3]. This reagent has been used as a general and microcolorimetric reagent for phenols[4-6] and also by Parthasarathi and Vijayasarathy[7,8] for the direct estimation of phenolic bodies (these include cane tannin and anthocyanin of the rind) in fresh cane juices. The phenolic bodies in 5 ml. aliquots of the suitably diluted fresh leaf tissue fluid were estimated by DSA using the procedure adopted for cane juices[7,8]. Two minutes were allowed for diazotization at the room temperature (22-27°C.) before the final development of colour by addition of alkali. The artificial standard adopted was 0·004*N*

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potassium dichromate solution which was itself standardized by comparison with the colour developed using a standard solution of phenol. The phenolic bodies were expressed in terms of phenol.

To eliminate possible interference by contact with metal during extraction of juice, the results obtained with the leaf tissue fluid extracted through the meat juice grinder were compared with values got with tissue fluid obtained by grinding the leaves in a mortar with quartz sand and a few drops of toluene. The results were the same within the limits of experimental error. The technique was further verified by the recovery of added phenol in both cases.

Results and discussion

In Table 1 are given the values for phenolic bodies present in leaves of healthy, spiked and partially spiked plants. The data reveal significant differences in the levels of the phenolic bodies in healthy and spiked leaves, the diseased leaves invariably showing a higher level of phenolic bodies than the healthy ones. A similar variation is also shown by healthy and spiked leaves collected from partially spiked plants. It is particularly noteworthy that this characteristic difference in healthy and diseased leaves was discernible even when there is a rapid decrease in the level of the phenolic bodies as the season advanced. Thus, the higher level of phenolic bodies appears to be a function distinctive of the metabolism of the spiked sandal leaves as it is independent of seasonal effects. This finding is in conformity with the results of Resuhr[9] who concluded that many external and internal symptoms shown by virus-infected plants are caused by disturbances of phenol or especially tannin metabolism of the plants.

TABLE 1 — PHENOLIC BODIES IN HEALTHY AND SPIKED SANDAL LEAVES

Date	Phenolic bodies (*g.*/100 *g. tissue fluid*) in			
	Healthy plants	Diseased plants	Partially diseased plants	
			Healthy part	Diseased part
	NURSERY (4 PLANTS)			
28 July 1960	1·40-2·72	—	—	—
17 Aug. 1960	0·29-0·57	—	—	—
12 Sept. 1960	0·15-0·20	—	—	—
	LAL BAGH (3 PLANTS)			
12 July 1960	1·16-1·72	—	—	—
16 Aug. 1960	0·26-0·33	—	—	—
16 Sept. 1960	0·14-0·15	—	—	—
	KENCHANAHALLY (9 PLANTS)			
11 July 1960	1·33-2·32	—	—	—
23 Aug. 1960	0·19-0·31	—	—	—
23 Sept. 1960	0·13-0·16	—	—	—
7 July 1960	—	3·73-6·60	—	—
2 Aug. 1960	—	1·64-1·95	—	—
16 Sept. 1960	—	0·55-0·87	—	—
22 Aug. 1960	—	—	0·35-0·85	0·92-1·09
24 Sept. 1960	—	—	0·25-0·42	0·47-0·57
	DOBBSPET (3 PLANTS)			
31 Aug. 1960	—	1·63-1·24	—	—

It was observed during the analyses that the colour developed by the leaf tissue fluid with DSA reagent was pure yellow in July, while it changed to reddish yellow in August and September in the case of all samples. This requires to be followed up. A possible explanation is a variation in the build-up of the chemical complex of which the phenolic body is only a constituent, or presumably, the formation of more complex phenols from simpler ones goes on as the season advances. It is known that the formation of polyphenols proceeds quite intensively in young plant tissues[10,11] and the polyphenols in newly forming leaves are largely in the form of simplest structural units[12], while the condensed tannin fraction[10] or products of higher order of condensation[12] predominate in the older tissues. The higher level of phenolic bodies, both in healthy and spiked leaves, in July, when there was active growth of the new foliage in all the plants, and the subsequent decrease of this level in August and September fit into this hypothesis.

Accumulation of phenolic bodies and sometimes of a particular phenolic body in the virus-infected plant tissues was noticed by some workers[13,14]. The higher level of phenolic bodies in spiked sandal leaves may be due to either a general increase in the different phenolic bodies present or to an increase in a particular phenolic body (bodies). A detailed investigation of the different phenolic bodies in the healthy and spiked sandal leaves would throw light on the dynamics of these bodies in spike disease as well as on their significance in the metabolism of the diseased plant. This study is being taken up. While the higher level of phenolic bodies in spiked leaves may possibly have connection with the increased respiration in them, no categorical assertion can be made and much less any conclusion drawn without further investigation.

The spiked plants exhibited vegetative activity only during the period of experimentation. In contrast, the healthy plants and the healthy branches of partially spiked plants manifested reproductive phases as well with the formation of green fruits during July and August followed by gradual ripening of the green fruits during September. Fluctuations in the level of phenolic bodies during the formation of flowers[15,16] and fruits[16] have been observed. In sandal, however, the seasonal fluctuation

of phenolic bodies in the healthy and diseased sandal leaves seems to be governed primarily by the vigour and stage of growth of the leaves.

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Short Communications

A Note on Roccella Found in Pondicherry

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The distribution of *Roccella montagnei* in Pondicherry area is reported. The earlier record regarding *Roccella fuciformis* in this locality seems to be incorrect.

ALTHOUGH the lichen flora of India has been reported to be very large and varied, we still have to make a systematic study regarding their distribution all over the country and proper identification. It has been recorded in Watt[1] and by Biswas[2] that *Roccella fuciformis* is found on mango trees in Pondicherry. Biswas[2] has also mentioned that this lichen is of medicinal value and is supposed to cure eczema.

In continuation of our earlier work[3] on Indian lichens and particularly *Roccella montagnei*[4,5], the lichen found on mango trees in and around Pondicherry has been collected and investigated for the chemical components present in the species The crystalline components of the samples collected during November and December 1959 and 1960 were found to be erythritol (3·0 per cent), erythrin (1·4 per cent) and roccellic acid (2·3 per cent). The total carotene content[6] was of a high order, 2·0 to 2·5 mg./g. of the air-dry lichen. The presence of ergosterol and orcinol could also be demonstrated. Picroroccellin, which had been reported[7,8] to be present in *R. fuciformis*, could not be isolated from the sample studied now. This suggested to us that the lichen found in Pondicherry (Fig. 1) is probably *R. montagnei*, agreeing with earlier samples examined by Seshadri and co-workers[4]. It resembles in all physical appearance the sample of *R. montagnei* investigated earlier[4].

The botanical identity of the sample as *R. montagnei* was also confirmed from Shri D. D. Awasthi, Department of Botany, Lucknow University, to whom our thanks are due. Hence, it is now reported that what had been recorded as *R. fuciformis* found on trees in Pondicherry seems to be incorrect and the distribution of *R. montagnei* in and around

Fig. 1 — *R. montagnei* found in Pondicherry

Pondicherry should be recognized. Here, it may also be mentioned that *R. fuciformis* is reported[8,9] to be commonly found on rocks.

Our thanks are due to Dr M. Balasubramaniam for the photograph of the lichen.

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Composition of Fat in Sewage as Affected by Aerobic & Anaerobic Treatment

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Aerobic treatment, unlike anaerobic treatment, of domestic sewage has been found to bring about greater oxidation of the fatty matter and formation of more ester fatty acids and synthesis of linolenic acid in the sludge.

IN view of the rapid removal of the fatty constituents of sewage by activated sludge[1] and the quality of sewage effluents in relation to their fat contents[2], a comparative study has been carried out on the fatty matter in the sludges formed under conditions similar to those in the activated sludge tank and septic tank, about which there is practically no information[3-7].

Samples of raw sewage from the same source were treated aerobically and anaerobically (33 litres for each treatment, the samples added at the same intervals over a period of 43 days). The effluents were taken out periodically and the sludges from the treatment systems were analysed for their fat composition[8], including the individual fatty acids which were determined by circular paper chromatographic method[9] (Table 1).

TABLE 1 — COMPOSITION OF FATTY MATTER IN SLUDGES AND EFFLUENTS FROM AEROBIC AND ANAEROBIC SYSTEMS

	Raw sewage	Aerobic system*: sludge	Anaerobic system	
			Sludge	Effluent
Total solids (oven dry), g.	18·07	4·12	1·76	12·70
Fatty matter, %	8·10	5·80	24·00	2·35
Fatty matter oxidized, %	—	81·85 (in the system)	50·67 (in the system)	
Saponifiables, % of total fat	80·30	82·80	70·00	80·90
Unsaponifiables, % of total fat	11·73	10·35	20·00	10·60
Non-ester fatty acids, % of total fatty acids	52·04	23·74	42·57	54·78
Ester-fatty acids, % of total fatty acids	47·96	76·26	57·43	45·22
Fatty acids, %				
Lauric	0·34	1·78	2·27	nil
Myristic	0·68	2·18	0·76	nil
Palmitic	32·77	21·08	22·35	27·73
Stearic	12·93	15·41	27·05	26·81
Oleic	47·67	19·07	25·23	8·98
Linoleic	2·70	34·50	17·63	32·14
Linolenic	nil	0·97	nil	nil

*The effluent from the aerobic system collected on the first, second and third days contained small, decreasing amounts of fatty matter (collectively 26 mg.); from the fourth day onwards until the end of the experiment, the effluent was free from fatty matter, as observed earlier[1,2].

It was of considerable interest to observe the formation of more ester fatty acids and the exclusive synthesis of linolenic acid in the aerobic sludge. The other observations include the greater oxidation of fat in the aerobic system and the presence of a much higher amount of fatty matter with a higher percentage of unsaponifiables in the anaerobic sludge.

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